J|A|C|S
COMMUNICATIONS

Published on Web 04/21/2007

Nanoparticle Self-Assembly Gated by Logical Proteolytic Triggers

Geoffrey von Maltzahn,[†] Todd J. Harris,[†] Ji-Ho Park,[‡] Dal-Hee Min,[†] Alexander J. Schmidt,[†] Michael J. Sailor,[‡] and Sangeeta N. Bhatia*[,†,§,||]

Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts, Department of Chemistry and Biochemistry, University of California, San Diego, California, MIT Electrical Engineering and Computer Science, Cambridge, Massachusetts, and Brigham & Women's Hospital, Boston, Massachusetts

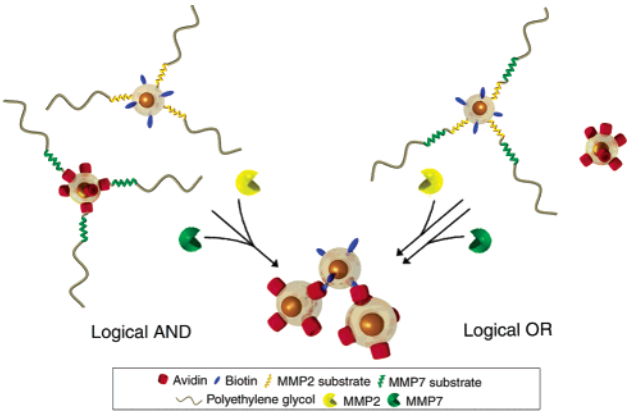
Received January 21, 2007; E-mail: sbhatia@mit.edu

The emergent electromagnetic properties of nanoparticle self-assemblies are being harnessed to build new medical and biochemical assays with unprecedented sensitivity. Nanoparticle (NP) assembly has been exploited to probe for a host of pathological inputs in vitro, including DNA,[1,2] RNA,[1] proteins,[3,4] viruses,[5] and enzymatic activity.[4,6,7] Typically, NP systems are designed to sense single molecular targets. While this methodology has been effective for in vitro applications, the future development of highly diagnostic in vivo sensors may benefit from the ability to simultaneously monitor multiple aspects of disease. In this report we describe a method whereby inorganic nanocrystals may perform Boolean logic operations using two inputs associated with cancer invasion (MMP2 and MMP7). Disperse, superparamagnetic Fe_3O_4 NPs are designed to coalesce in response to logical AND or OR functions. In either system, NP self-assembly amplifies the *T*2 relaxation rate of hydrogen protons, enabling remote, MRI-based detection of logical operations. In the future, we believe that these sensors may be optimized to monitor a diversity of inputs in vitro and in vivo.

Logical operations were designed to analyze inputs of two matrix-metalloproteinases, a family of at least 26 secreted and membrane-bound proteases that have been studied extensively for their role in cancer.[8] In particular, matrix-metalloproteinase-2 (MMP2), is overexpressed in many cancers, including breast cancers, and is an indicator of cancer invasiveness, metastasis, angiogenesis, and treatment efficacy.[9] MMP7, a protease with broader substrate specificity, is thought to facilitate early stages of mammary carcinoma progression.[10] In tissues excised from breast cancer patients, both MMP-2 and MMP-7 were expressed at statistically higher levels in carcinogenic than in normal breast tissues,[11] highlighting their potential utility as dual markers of neoplastic inception. We demonstrate using dynamic light scattering (DLS), fluorescence, and MRI that logical sensors can probe samples for the presence of both MMP2 *and* MMP7 (AND function) or for the presence of either MMP2 *or* MMP7 (OR function).

To synthesize both sensor types, two kinds of NPs were initially engineered: one with a tethered ligand (biotin) and the other with its receptor (neutravidin). These particles were stable separately, but aggregated readily when combined. We sought to completely mask these groups by attachment of peptide-polyethyleneglycol (PEG) conjugates to conditionally prevent assembly. Previously, we demonstrated that two 10 kDa PEG-modified NPs could mutually deter each other's binding.[6] Here, by increasing the polymer weight to 20 kDa, we demonstrate that modification of only one NP can completely inhibit the binding of an unmodified NP (Figure S2; Figure 2). Accordingly, we reasoned that by linking polymers to each particle via unique protease substrates, assembly could be restricted to occur only in the presence of both enzymes (logical AND) (Scheme 1). Furthermore, by anchoring polymers to only the ligand NP with a tandem peptide substrate (containing both enzyme cleavage motifs in series), we sought to actuate assembly in the presence of either *or* both of the enzyme inputs (logical OR) (Scheme 1).

Scheme 1. Schematic Representation of Logical Nanoparticle Sensors[a]



[a] Self-assembly is gated to occur in the presence of MMP2 and MMP7 (logical AND) (left) or in the presence of either or both proteases (logical OR) (right) by attachment of protease-removable polyethylene glycol polymers

Peptide PEG polymers were synthesized by reacting the peptide N-terminus (or lysine residue for OR tandem peptide) with amine-reactive 20 kDa methoxy-PEG-succimidyl α-methylbutanoate (see Supporting Information for details). Cysteine residues were incorporated at the C-terminus of peptides to allow oriented attachment of substrate polymers to NPs, and TAMRA fluorochromes were incorporated distally to fluorescently monitor polymer removal. To begin AND NP synthesis, ligand particles were shielded with an MMP2 (Gly-Pro-Leu-Gly-Val-Arg-Gly)[12,13] substrate-polymer and receptor particles were shielded with an MMP7 (Val-Pro-Leu-Ser-Leu-Thr-Met)[14] substrate-polymer. Enzyme specificity for these sequences was assessed by monitoring TAMRA fluorescence increase in the presence of various enzymes. Specific enzyme−substrate pairs rapidly increased TAMRA fluorescence as cleaved substrate fluorochromes diffused away from the quenching iron oxide cores (Figure S1). Nonspecific pairs, including human thrombin, factor Xa, and serum, showed little fluorescence increase. We next examined each enzyme's ability to actuate assembly of peptide-shielded particles in the presence of their unmodified cognate particles. Specific enzyme−substrate pairs rapidly catalyzed the formation of nano- and microassemblies when incubated with unmodified cognate NPs, while nonspecific pairs negligibly affected

[†] Harvard-MIT Division of Health Sciences and Technology.
[‡] University of Californiia.
[§] MIT Electrical Engineering and Computer Science.
[||] MIT/Brigham & Women's Hospital.

10.1021/ja070461l CCC: $37.00 © 2007 American Chemical Society

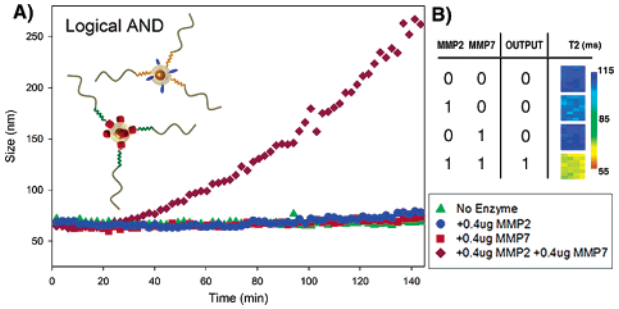


Figure 1. Logical AND: (a) Hydrodynamic radius in dynamic light scattering is only increased in the presence of both MMP2 and MMP7; either or none is insufficient to actuate assembly (40 ug Fe/mL). (b) Assemblies express AND logic in MRI. *T*2 relaxation decreases ~30% in 3 h following addition of MMP2 and MMP7, with nominal changes following addition of either enzyme alone (7.5 ug Fe/mL).

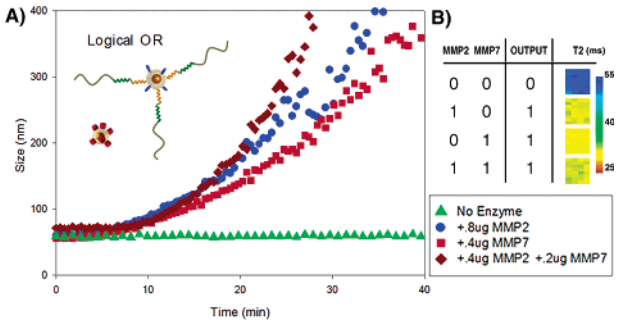


Figure 2. Logical OR: (a) Population hydrodynamic radius is increased in the presence of either or both MMP2 and MMP7 (40 ug/mL Fe). (b) MRI visualization of logical function demonstrates ~40% enhancement in *T*2 relaxation in the presence of either MMP2 or MMP7 or both enzymes (15 ug/mL Fe).

population size (Figure S2). To investigate the relationship between peptide polymer removal and NP assembly, biotin-MMP2-PEG enzyme reactions were quenched at various timepoints (Figure S3) and incubated with receptor NPs. Following removal of ~50% of NP polymers, assembly begins, eventually revealing sufficient biotin to direct NP precipitation.

To create a logical AND NP system, MMP2-PEG ligand particles and MMP7-PEG receptor particles were combined. Here, in the presence of either protease alone, assembly of NPs was prohibited by the PEG polymers remaining on the cognate particle (Figure 1a). In the presence of both proteases, however, NP self-assembly began and the population hydrodynamic radius increased 5-fold over 3 h in DLS (Figure 1a). Further, nanoparticles were able to express AND logic in *T*2 relaxation changes, mapped using a 4.7 T Bruker MRI. In the presence of both enzymes, *T*2 relaxation is enhanced by ~30% as compared to samples with either enzyme alone or none (Figure 1b). This enhancement is comparable to published magnetic relaxation sensors[1,5,6] and occurs at MMP2 concentrations that mimic tumor activity levels in vivo (4 ug MMP2/mL = 221 U/mL vs 435 U/g in vivo[13]).

A second system was constructed to actuate assembly in the presence of either of two proteolytic inputs (logical OR). Here, only the particles containing the ligand were masked with peptide-linked polymers. To allow either enzyme to actuate assembly, a tandem MMP2−MMP7 peptide substrate was synthesized, containing both cleavage motifs in series (separated by an aminohexanoic acid spacer). Hydrodynamic radii increased more than 5-fold in the presence of either or both enzymes, indicating proper OR function (Figure 2a). Accordingly, OR NP *T*2 relaxation decreases ~40% in the presence of either or both enzymes, as compared to samples with no enzyme (Figure 2b).

In conclusion, we have demonstrated the synthesis of NPs that use Boolean logic to simultaneously monitor multiple biological processes associated with tumorigenesis. In the future, we anticipate that logical NP switches may enable more informative imaging of neoplastic transformation in optically opaque samples both in vitro and in vivo. The modular design of these logical NP sensors can be applied to other enzymatic triggers, complementary ligand/receptor pairs, or NP cores (semiconductor, plasmonic). Looking further, logical NP switches may enable specific localization of the processes underlying malignant transformation in vivo, as proteolytically assembled beacons in sites of neoplastic inception. Such interstitial assembly may amplify the retention of particles (by mechanical entrapment in the tumor interstitium) and enhance MRI visualization of diagnostic logic functions.

Acknowledgment. We thank Erkki Ruoslahti for helpful discussions and critical review of this work. Financial support from NIH (BRP: R01CA124427-01), NIH/NCI (U54 CA119349, U54 CA119335), Packard Fellowship (1999-1453), Whitaker Foundation Graduate Fellowship, and NSF Graduate Fellowship (G.v.M.).

Supporting Information Available: DLS assembly of AND components, fluorescence enzyme specificity tests, partial cleavage NP assembly data, and detailed synthesis/methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Perez, J. M.; Josephson, L.; O'Loughlin, T.; Hogemann, D.; Weissleder, R. *Nat. Biotechnol.* **2002**, *20* (8), 816−820.

(2) Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff, J. J. *Nature* **1996**, *382* (6592), 607−609. Elghanian, R.; Storhoff, J. J.; Mucic, R. C.; Letsinger, R. L.; Mirkin, C. A. *Science* **1997**, *277* (5329), 1078−1081.

(3) Georganopoulou, D. G.; Chang, L.; Nam, J. M.; Thaxton, C. S.; Mufson, E. J.; Klein, W. L.; Mirkin, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (7), 2273−2276.

(4) Perez, J. M.; Simeone, F. J.; Tsourkas, A.; Josephson, L.; Weissleder, R. *Nano Lett.* **2004**, *4* (1), 119−122.

(5) Perez, J. M.; Simeone, F. J.; Saeki, Y.; Josephson, L.; Weissleder, R. *J. Am. Chem. Soc.* **2003**, *125* (34), 10192−10193.

(6) Harris, T. J.; von Maltzahn, G.; Derfus, A. M.; Ruoslahti, E.; Bhatia, S. N. *Angew. Chem., Int. Ed.* **2006**, *45* (19), 3161−3165.

(7) Wang, Z.; Levy, R.; Fernig, D. G.; Brust, M. *J. Am. Chem. Soc.* **2006**, *128* (7), 2214−2215. Zhao, M.; Josephson, L.; Tang, Y.; Weissleder, R. *Angew. Chem., Int. Ed.* **2003**, *42* (12), 1375−1378.

(8) Chang, C.; Werb, Z. *Trends Cell Biol.* **2001**, *11* (11), S37−S43. Edwards, D. R.; Murphy, G. *Nature* **1998**, *394* (6693), 527−528.

(9) Stearns, M. E.; Wang, M. *Cancer Res.* **1993**, *53* (4), 878−883. Talvensaari-Mattila, A.; Paakko, P.; Turpeenniemi-Hujanen, T. *Br. J. Cancer* **2003**, *89* (7), 1270−1275. Davidson, B.; Goldberg, I.; Kopolovic, J.; Lerner-Geva, L.; Gotlieb, W. H.; Ben-Baruch, G.; Reich, R. *Gynecol. Oncol.* **1999**, *73* (3), 372−382. Kanayama, H.; Yokota, K.; Kurokawa, Y.; Murakami, Y.; Nishitani, M.; Kagawa, S. *Cancer* **1998**, *82* (7), 1359−1366. Fang, J. M.; Shing, Y.; Wiederschain, D.; Yan, L.; Butterfield, C.; Jackson, G.; Harper, J.; Tamvakopoulos, G.; Moses, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (8), 3884−3889. Ratnikov, B. I.; Deryugina, E. I.; Strongin, A. Y. *Lab. Invest.* **2002**, *82* (11), 1583−1590. Giannelli, G.; FalkMarzillier, J.; Schiraldi, O.; StetlerStevenson, W. G.; Quaranta, V. *Science* **1997**, *277* (5323), 225−228.

(10) Rudolph-Owen, L. A.; Chan, R.; Muller, W. J.; Matrisian, L. M. *Cancer Res.* **1998**, *58* (23), 5500−5506. Hulboy, D. L.; Gautam, S.; Fingleton, B.; Matrisian, L. M. *Oncol. Rep.* **2004**, *12* (1), 13−17.

(11) Pacheco, M. M.; Mourao, M.; Mantovani, E. B.; Nishimoto, I. N.; Brentani, M. M. *Clin. Exp. Metastasis* **1998**, *16* (7), 577−585.

(12) Seltzer, J. L.; Akers, K. T.; Weingarten, H.; Grant, G. A.; McCourt, D. W.; Eisen, A. Z. *J. Biol. Chem.* **1990**, *265* (33), 20409−20413.

(13) Bremer, C.; Tung, C. H.; Weissleder, R. *Nat. Med.* **2001**, *7* (6), 743−748.

(14) Turk, B. E.; Huang, L. L.; Piro, E. T.; Cantley, L. C. *Nat. Biotechnol.* **2001**, *19* (7), 661−667.

JA070461L